ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1995, p. 2606–2609 Vol. 39, No. 12
0066-4804/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Potent Antimalarial Activity of the Alkaloid Nitidine, Isolated from a Kenyan Herbal Remedy

D. M. N. GAKUNJU,[1] E. K. MBERU,[2] S. F. DOSSAJI,[3] A. I. GRAY,[4] R. D. WAIGH,[4]*
P. G. WATERMAN,[4] AND W. M. WATKINS[2,5,6]

Ministry of Health,[1] The Wellcome Trust Research Laboratories,[2] The National Museums of Kenya,[3] and Kenya Medical Research Institute,[6] Nairobi, Kenya; Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow, Scotland[4]; and Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool, United Kingdom[5]

Received 19 June 1995/Returned for modification 22 August 1995/Accepted 23 September 1995

Bioassay-guided fractionation of extracts of *Toddalia asiatica*, a plant used by the Pokot tribe of Kenya to treat fevers, has yielded the alkaloid nitidine as the major antimalarial component. Fractions containing nitidine have in vitro 50% inhibitory concentrations against *Plasmodium falciparum* in the range of 9 to 108 ng/ml for a range of chloroquine-susceptible and -resistant strains. The results show a lack of cross-resistance between chloroquine and nitidine.

Although malaria is in principle preventable and curable, in practice the majority of the population at risk cannot pay for modern treatment and the disease remains a major cause of childhood mortality in the developing world. Recent results with the vaccine sP*f*66 show a mean protective effect of only 30%, with very wide confidence limits (1). The rapid spread of resistance to chloroquine and related quinoline-based antimalarial agents has greatly increased the risk of malaria to many rural populations, so that there is an urgent need for affordable treatment. One possible source of such treatment lies in the traditional herbal remedies used by ethnic groups, but treatment with these remedies has suffered from a number of deficiencies. Diagnosis is often a problem, identification of plant material may be insecure, and the chemical content of extracts may vary considerably. We have set out to overcome these barriers to effective treatment by systematically evaluating the effectiveness of some of the remedies used by an ethnic group of Kenyan farmers.

The Pokot traditionally inhabit a highland plateau, west of the Rift Valley in Kenya. However, the present study concerned a group of Pokot who live on Ol Ari Nyiro Ranch, Laikipia, Kenya, a plateau to the east of the Rift Valley, and who have previously been described in some detail (5).

In interviews, the Pokot herbalist Cheptosai Selale, who is 90 years old, described the use of 26 plants for the treatment of malaria and fever, of which only 14 were available at the time of collection (see Table 1). Two of the 14 plants produced extracts with significant antimalarial activity against laboratory strains of *Plasmodium falciparum*. Bioassay-guided fractionation of the extract from *Toddalia asiatica* gave a pure alkaloid, nitidine, which has potentially useful antimalarial activity.

MATERIALS AND METHODS

Plant material. Plant material was collected from Ol Ari Nyiro Ranch and was botanically authenticated by Christine Kabuye and Joshua Muasya of the Herbarium, National Museums of Kenya, where voucher specimens were deposited. Information provided by the herbalist included the required part of the plant, the precise locality for collection, and the time when curative potency was maximal. Plant material for study was dried at room temperature, pulverized, and stored dry in plastic bags until extracts were obtained.

Preparation of aqueous crude extracts for preliminary analysis. Aqueous crude extracts (10%) were prepared in a manner analogous to that used by the herbalist. Powdered plant material (10 g) was weighed into a beaker, 100 ml of distilled water was added, and the mixture was brought to the boil, cooled, and left overnight to macerate. Extracts were filtered through no. 1 Whatman filter paper, and the volume of filtrate was noted. Solutions were then sequentially filtered through membrane filters (Millipore, Harrow, United Kingdom) with pore sizes of 0.8, 0.45, and finally, 0.22 μm, and the final volume of filtrate was noted. Aliquots of 2 ml were freeze-dried to determine the concentration (in grams per milliliter) of solute in each extract before freeze-drying the remaining crude extract.

Preparation of extracts in a form suitable for the in vitro test required consideration of both the aqueous solubility and the sterility of each extract. Each compound was redissolved in a measured volume of either water or ethanol, which was then further diluted with its counterpart to yield a 70% ethanol–30% water mixture containing a known concentration of extract. This solution was allowed to stand at room temperature for 30 min to sterilize the solution. Further dilutions were made with *P. falciparum* culture medium (RPMI 1640 medium containing 25 mmol of HEPES [*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid] buffer per liter, 25 mmol of sodium bicarbonate per liter, and 10% normal, pooled human serum). Final dilutions on the in vitro test plate contained <0.1% ethanol, which is not inhibitory to the parasite in this system (3), although controls were included in each test series.

Extraction and fractionation of *T. asiatica*. Air-dried, pulverized root bark was first extracted twice with dichloromethane (500 g to 1 liter) by overnight maceration to remove fats. The residue was then extracted twice with cold methanol (500 g to 1 liter), and the filtrates were combined and dried in vacuo. The alkaloids in the crude methanolic fraction were concentrated by the acid-base procedure (6), and the resulting chloroform fraction was further fractionated by vacuum-liquid column chromatography with silica gel 60 and a solvent system of increasing polarity (petrol, chloroform, methanol). Fifteen fractions were collected, dried in vacuo, and stored at 4°C until they were tested.

For in vitro tests, approximately 10 mg, weighed to 0.01 mg, was dissolved in 70% ethanol and was allowed to stand for 30 min to sterilize the solution. The ethanolic solution was further diluted in culture medium to an appropriate test concentration. A stock solution of authentic nitidine was prepared in a similar manner.

In vitro antimalarial activity tests. The antimalarial activity test was based on previously reported methods (3, 10). Laboratory cultures of *P. falciparum* were maintained by standard methods. For the test, 25-μl aliquots of culture medium were added to all of the wells of a 96-well flat-bottom microculture plate (Sterilin, Teddington, United Kingdom). Aliquots of the test solutions of 25 μl were added, in duplicate, to the first wells, and a Titertek motorized hand diluter (Flow Laboratories, Uxbridge, United Kingdom) was used to make serial 2-fold dilutions of each sample over a 64-fold concentration range. Aliquots of 200 μl of a 1.5% (vol/vol) suspension of parasitized erythrocytes in culture medium (0.4% parasitemia; growth rate, >threefold per 48 h) were added to all test wells. Parasitized and nonparasitized erythrocytes and solvent controls were incorporated into all tests. The plates were incubated at 37°C in a gas mixture of 3% CO_2–5% O_2–92% N_2. After 48 h each well was pulsed with 25 μl of culture medium containing 0.5 μCi of [^{3}H]hypoxanthine and the plates were incubated for a further 18 h. The contents of each well were then harvested onto glass fiber filters, washed thoroughly with distilled water, and dried, and the radioactivity (in counts per minute) was measured by liquid scintillation. The regression function log cpm $= a + b \times$ (log drug concentration), where *a* is the *y* intercept and *b* is

* Corresponding author. Phone: 01415524400. Fax: 01415526443.

TABLE 1. In vitro activities of aqueous extracts from Kenyan plants used by the Pokot herbalists, Laikipia, Kenya, against three Kenyan strains of *P. falciparum*

Plant species	Family	Plant part	IC_{50} (μg/ml)[a]		
			M24	K67	ENT 7
Canthium phyllanthoideum	*Rubiaceae*	Stem bark	208	360	540
Olinia usambarensis	*Oliniaceae*	Stem bark	204	130	380
Pittosporum vividiflorum	*Pittosporaceae*	Stem bark	80	30	170
Aspilia mossambicensis	*Compositae*	Leaves	406	730	720
Gardenia jovis-tonantis	*Rubiaceae*	Root bark	820	880	1,750
Toddalia asiatica	*Rutaceae*	Root bark	20	5	20
Rhoicissus tridentata	*Vitaceae*	Whole tuber	100	40	70
Turraea mombassana	*Meliaceae*	Whole root	150	90	100
Heteromorpha trifolia	*Umbelliferae*	Stem bark	475	370	580
Shrebera alata	*Oleaceae*	Stem bark	627	160	380
Maytenus arbutifolia	*Celastraceae*	Whole root	4	4	10
Rhamnus staddo	*Rhamnaceae*	Root bark	520	490	740
Scutia myrtina	*Rhamnaceae*	Root bark	40	240	320
Cucumis aculeatus	*Cucurbitaceae*	Whole fruit	60	30	30

[a] Each IC_{50} is the mean of at least two in vitro tests carried out on different days.

the slope, was calculated by using duplicate points above and below the midpoint of the counts per minute between the parasitized and nonparasitized controls. The concentration causing 50% inhibition of radioisotope incorporation (IC_{50}) was determined by interpolation as described previously (13). This method gives a value equal to 100.4% ± 10.6% (mean ± standard deviation) of the value estimated by nonlinear regression analysis (14). Statistical differences between means for small populations were examined by Student's *t* test.

RESULTS

The results of tests with crude extracts against three strains of *P. falciparum* are presented in Table 1. Only the extracts of the root bark of *T. asiatica* and the whole root of *Maytenus arbutifolia* showed significant activity in this test. Although this preliminary screen does not preclude the possibility that the other extracts may be active antimalarial agents by the oral route, subsequent work focused on the plants with demonstrable activity. Of these, *M. arbutifolia* was known as a source of cytotoxic agents such as maytansine (7) and produced extracts of great complexity; the present study therefore concentrated on *T. asiatica*, which has also been intensively studied (2) but which proved to be much more amenable to bioassay-guided fractionation.

Sequential extracts from *T. asiatica* showed different levels of antimalarial activity (Table 2). The methanolic extract showed the highest activity, with a mean 50% inhibitory dose of 0.98 μg/ml for the chloroquine-susceptible strains K67, K39, and M24. Acid-base extraction of the methanolic extract (6.08 g) yielded 0.1893 g (3.1%) of petroleum ether-soluble extract, 0.0246 g (0.4%) of acidic chloroform extract, and 0.99 g (16.3%) of basic chloroform extract. The highest potency was observed in the basic chloroform extract coded TA106(13), which had an IC_{50} of <1 μg/ml. This extract was further fractionated by chromatography on silica by circular preparative thin-layer chromatography by eluting with ethyl acetate-methanol-water-ammonia (100:17:13:3), which gave a single yellow compound at a 10-mg yield from 500 g of powdered root bark. The UV spectrum showed absorbances at 235, 275, 293, 303, 332, and 380 nm, which is similar to the data for nitidine (18). Comparison with an authentic sample of nitidine (compound 1) both chromatographically and spectroscopically (mass spectrometry, infrared spectroscopy, and high-field nuclear magnetic resonance) proved the identity of the unknown compound.

TABLE 2. In vitro activities of extract fractions from *T. asiatica* against *P. falciparum*

Extract	IC_{50} (μg/ml)[a]		
	K67	K39	M24
Dichloromethane	11.93	7.65	18.05
Methanol	0.7	0.78	1.46
Water	21.14	31.67	740.0
Active fraction		0.04	
Nitidine		0.045	
Dihydronitidine		1.03	
Chloroquine		0.004	

[a] Each IC_{50} is the mean of at least two in vitro tests carried out on different days. For the V1/s strain, IC_{50}s were 0.057 μg/ml for chloroquine and 0.941 μg/ml for dihydronitidine.

The electron impact mass spectrum gave a molecular ion at *m/z* 348 with the formula $C_{21}H_{18}NO_4$ by accurate mass measurement. The base peak in the spectrum, at *m/z* 333, is attributable to the loss of a methyl group. The ^1H nuclear magnetic resonance spectrum showed seven aromatic protons, five singlets at δ 9.61, 8.27, 8.22, 7.80, and 7.59, as well as two doublets (J = 9.2 Hz) centered at δ 8.72 and 8.21. The methylenedioxy group resonated as a two-proton singlet at δ 6.27, and there were three signals for methyl groups at δ 4.93, 4.26, and 4.11, with that at the lowest field being attributable to the *N*-methyl. Comparison of the active fraction with an authentic sample of nitidine by thin-layer chromatography on silica with ethyl acetate-methanol-water-ammonia (100:17:13:3) as the mobile phase gave spots with R_f values of 0.25, having the same color and response to UV light.

Subsequent in vitro studies examined the chemosensitivity of additional laboratory strains of *P. falciparum*, characterized for chloroquine susceptibility, to the active fraction and authentic nitidine by using three chloroquine-resistant and four chloroquine-susceptible strains. Each IC_{50} measurement was determined in duplicate as described above, and each test was performed in duplicate; Table 3 compares the activities of the active fraction, authentic nitidine, and chloroquine against groups of chloroquine-susceptible and chloroquine-resistant laboratory strains of *P. falciparum* in vitro.

DISCUSSION

The antimalarial activities of a number of Tanzanian plants, many of which also occur in Kenya, have been reviewed by

TABLE 3. In vitro chemosensitivity of *P. falciparum* isolates to an alkaloidal fraction [TA106(13)] from *T. asiatica*, nitidine, and chloroquine

Isolate	Chloroquine susceptibility	IC_{50} (ng/ml)		
		Chloroquine	TA106(13)	Nitidine
UPA	Susceptible	16.0	56.0	67.0
K39	Susceptible	4.4	40.3	45.1
SL/D6	Susceptible	6.6	29.3	76.0
HB3	Susceptible	5.9	40.4	73.6
		8.22 ± 5.3	41.5 ± 11.0	65.4 ± 14.1
ItD12	Resistant	65.9	9.2	42.0
FCR3	Resistant	47.4	37.5	165
FCB	Resistant	28.4	108.0	47.5
		47.2 ± 15.3	51.6 ± 41.5	84.8 ± 56.7

[a] Each IC_{50} is the mean of at least two in vitro tests carried out on different days.

Weenen et al. (17). Only 2 of 49 plants from that review (*Gardenia jovis-tonantis* and *T. asiatica*) were also used by the Kenyan Pokot herbalists. It is of interest that *T. asiatica*, one of the two plants producing active extracts in our study, is also used by herbalists in Tanzania and has been reported to produce extracts with moderate in vitro activity against the parasite (16).

Nitidine is a well-known cytotoxic agent which has received considerable attention as a potential anticancer drug following the discovery of potent antileukemic activity in mice (9). Clinical trials were eventually terminated for reasons which are unclear but which may have been due to host toxicity. Nitidine is a quaternary salt, charged at all pH values, and therefore, in theory it is prone to poor absorption from the gastrointestinal tract, but it is capable of existing as the tautomeric pseudobase (compound 2), which would be absorbable (Fig. 1). It was also possible that dihydronitidine (compound 3), identified as a minor component of *T. asiatica*, could account for the in vivo antimalarial activity. Dihydronitidine is not charged and is therefore able to pass membranes; inside cells it could be oxidized to release nitidine. However, dihydronitidine was only weakly antimalarial (Table 2), suggesting that it is not a prodrug for nitidine in our test system.

Although the crude aqueous extract, with an IC_{50} of 20 μg/ml, exhibited comparatively weak activity, when we compared both the active fraction of *T. asiatica* and authentic nitidine against *P. falciparum* strains with different responses to chloroquine, it was apparent that the alkaloid exerted potent activity (Table 3). The active fraction, with a mean IC_{50} of 51.6 ng/ml for chloroquine-resistant strains, is more active than quinine against Kenyan parasites (IC_{50}, ca. 120 ng/ml) (11).

The isolation procedure concentrated on obtaining a sample pure enough for identification. No attempt was made to determine the total amount of nitidine in the root bark. Such a determination would be complicated by the known instability of nitidine in alkaline solution, whereby the pseudobase form (compound 2) disproportionates to give the dihydro form (compound 3) and the 6-keto form known as oxynitidine, which is not charged and which is unlikely to be biologically active. Our experience with other species of the family *Rutaceae* suggests that there are unlikely to be major amounts of alkaloids similar to nitidine in the root bark of *T. asiatica*, nor was there chromatographic evidence for other major active constituents. This does not preclude the existence of chemically unrelated compounds in the extracts with direct or synergistic activity; however, from the data in Tables 1 and 2, it may be seen that nitidine is two orders of magnitude more potent than the crude extract. Given the inevitable losses in the extraction process, there is little doubt that nitidine accounts for a high proportion of the observed activity.

The mean IC_{50} of chloroquine for the chloroquine-resistant isolates of 47.2 ng/ml (189 nmol/liter) exceeds the threshold MIC of 114 nmol/liter used to define chloroquine-resistant infections in Kenya and elsewhere (12, 15), confirming the chemosensitivity of the isolates used. We found a significant difference in the mean IC_{50} of chloroquine between those for chloroquine-resistant isolates and those for chloroquine-susceptible isolates ($P < 0.05$) but not between the IC_{50}s of the active fraction or nitidine ($P > 0.05$ for both comparisons). The resistance of *P. falciparum* to chloroquine is now a major health problem in Kenya, as in many parts of Africa, and these data suggest a potential role for *T. asiatica* extracts in the treatment of chloroquine-resistant falciparum malaria. Further work is needed to confirm the activity of this alkaloidal extract against chloroquine-resistant parasites and to determine more

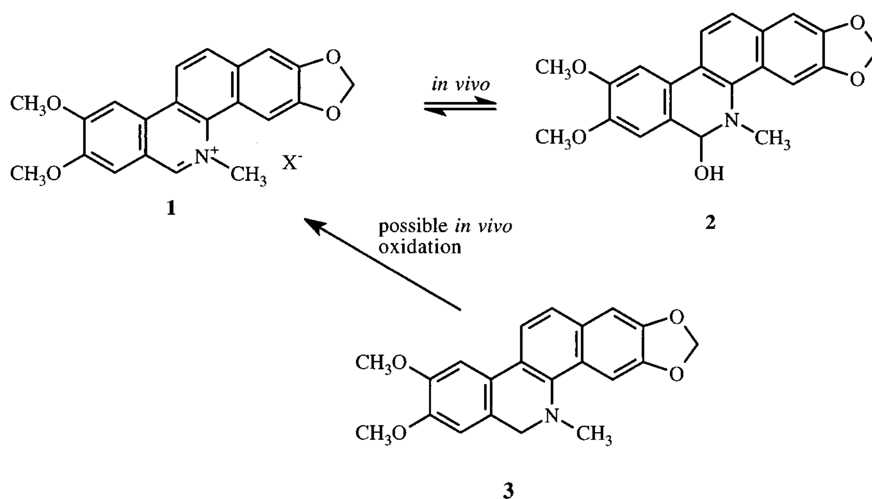

FIG. 1. Chemical structures of nitidine (compound 1), the tautomeric pseudobase form (compound 2), and the possible prodrug 5,6-dihydronitidine (compound 3).

exactly the compositions of the extracts prepared by the Pokot herbalists.

It is possible that long-term consumption of *T. asiatica* may be deleterious, given the cytotoxic effects of nitidine, although short-term use to treat a life-threatening disease may, in future, be supportable on risk-benefit analysis and in view of the availability of the plant and its low cost. Nitidine and its sister alkaloid fagaronine have recently been shown to be topoisomerase inhibitors (4, 8); thus, it is possible that the antimalarial action is mediated through the inhibition of the parasite enzyme. If this is the case, analogs which bind preferentially to the parasite enzyme may represent a source of new antimalarial drugs, and we are pursuing this. The absence of cross-resistance between nitidine-containing extracts and chloroquine may be of particular significance in the potential use of these compounds as drugs for antimalarial treatment.

ACKNOWLEDGMENTS

We thank the Director of Medical Services, Ministry of Health, Nairobi, Kenya, for financial support and for permission to publish these results. We gratefully acknowledge a grant from the Gallman Memorial Foundation and the personal assistance provided by the director, K. Gallman.

The present study would not have been possible without the willing assistance of the Pokot herbalists Cheptosai Selale (Mama Rangeta), her daughter Saline Kirabu, and Esta Boliamoi. We are also grateful to Christine Kabuye and Joshua Muasya of the herbarium, National Museums of Kenya. E.K.M. and W.M.W. are grateful to the Wellcome Trust, London, United Kingdom, for personal and grant support.

REFERENCES

1. **Alonso, P. L., T. Smith, J. R. M. Armstrong-Schellenberg, H. Masanja, S. Mwankusye, H. Urassa, I. De Bastos Azevedo, J. Chongela, S. Kobero, C. Menendez, N. Hurt, M. C. Thomas, E. Lyimo, N. A. Weiss, R. Hayes, A. Y. Kitua, M. C. Lopez, W. L. Kilama, T. Teuscher, and M. Tanner.** 1994. Randomised trial of efficacy of SPf66 vaccine against *Plasmodium falciparum* malaria in children in Southern Tanzania. Lancet **344:**1175–1181.
2. **Chen, I.-S., I.-L. Tsai, S.-J. Wu, W.-S. Sheen, T. Ishikawa, and H. Ishii.** 1993. Toddaquinoline from Formosan *Toddalia asiatica*. Phytochemistry **34:**1449–1451.
3. **Desjardins, R. E., C. J. Canfield, J. D. Haynes, and J. D. Chulay.** 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob. Agents Chemother. **16:**710–718.
4. **Fang, S. D., L. K. Wang, and S. M. Hecht.** 1993. Inhibitors of DNA topoisomerase I isolated from the roots of *Zanthoxylum nitidum*. J. Org. Chem. **58:**5025–5027.
5. **Gallman, K.** 1991. I dreamed of Africa. Viking Penguin, New York.
6. **Harborne, J. B.** 1984. Phytochemical methods, 2nd ed. Chapman & Hall, London.
7. **Komoda, Y., and T. Kishi.** 1980. Anticancer agents based on natural products. Academic Press, Inc., New York.
8. **Larsen, A. K., L. Grondard, J. Couprie, B. Desoize, L. Comoe, J. C. Jardillier, and J. R. Riou.** 1993. The antileukaemic alkaloid fagaronine is an inhibitor of DNA topoisomerases I and II. Biochem. Pharmacol. **46:**1403–1412.
9. **Messmer, W. M., M. Tin-Wa, H. H. S. Fong, C. Bevelle, N. R. Farnsworth, D. J. Abraham, and J. Trojanek.** 1972. Fagaronine, a new tumor inhibitor isolated from *Fagara zanthoxyloides* Lam. (Rutaceae). J. Pharm. Sci. **61:**1858.
10. **O'Neill, M. J., D. H. Bray, P. Boardman, J. D. Phillipson, and D. C. Warhurst.** 1985. Plants as sources of antimalarial drugs. Part 1. *In vitro* test method for the evaluation of crude extracts from plants. Planta Medica **50:**394–397.
11. **Pasvol, G., C. R. J. C. Newton, P. A. Winstanley, W. M. Watkins, N. M. Peshu, J. B. O. Were, K. Marsh, and D. A. Warrell.** 1991. Quinine treatment of severe falciparum malaria in African children: randomized comparison of three regimens. Am. J. Trop. Med. Hyg. **45:**702–713.
12. **Rieckman, K. H., L. J. Sax, G. H. Campbell, and J. E. Mrema.** 1978. Drug sensitivity of *Plasmodium falciparum*: an *in vitro* microtechnique. Lancet **311:**22–23.
13. **Sixsmith, D. G., W. M. Watkins, J. D. Chulay, and H. C. Spencer.** 1984. *In vitro* antimalarial activity of tetrahydrofolate dehydrogenase inhibitors. Am. J. Hyg. Trop. Med. **33:**772–776.
14. **Watkins, W. M., D. G. Sixsmith, and J. D. Chulay.** 1984. The activity of proguanil and its metabolites cycloguanil and *p*-chlorophenylbiguanide against *Plasmodium falciparum in vitro*. Ann. Trop. Med. Parasitol. **78:**273–278.
15. **Watkins, W. M., D. G. Sixsmith, H. C. Spencer, D. A. Boriga, D. M. Kariuki, T. Kipingor, and D. K. Koech.** 1984. Effectiveness of amodiaquine as treatment for chloroquine-resistant *Plasmodium falciparum* infections in Kenya. Lancet **323:**357–359.
16. **Watt, J. M., and M. G. Breyer-Brandwijk.** 1962. Medicinal and poisonous plants of southern and eastern Africa, 2nd ed. Livingstone, Edinburgh.
17. **Weenen, H., M. H. H. Nkunya, D. H. Bray, L. B. Mwasumbi, L. S. Kinabo, and V. Kilimali.** 1990. Antimalarial activity of Tanzanian medicinal plants. Planta Medica **56:**368–370.
18. **Zee-Cheng, K.-Y., and C. C. Cheng.** 1973. Synthesis of 5,6-dihydro-6-methoxynitidine and a practical preparation of nitidine chloride. J. Heterocycl. Chem. **10:**85–88.